APOPTOSIS SIGNAL-REGULATING KINASE 1: AS A NOVEL REGULATOR OF THE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE STRESS-SIGNALING CASCADE

by
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Abstract

GAPDH is a classical glycolytic enzyme that has recently been demonstrated to play critical roles outside of glycolysis. These multifunctional roles include the ability for GAPDH to function in DNA repair, membrane fusion, cytoskeletal dynamics, and cell death. These acquired multifunctional roles of are largely dictated by its subcellular localization or by posttranslocation modifications that push GAPDH from the cytoplasm to various subcellular niches. Among several posttranslational modifications, oxidation of GAPDH at C150 is one of the most well characterized modifications that have established GAPDH as a sensor of oxidative stress. In the presence of oxidative stress, oxidation of GAPDH mediates its translocation to the nucleus, via Siah1, where it mediates a stress response, cell death or cellular dysfunction in a "gain-of-toxic function" manner. Recently, an increasing number of studies have demonstrated that nuclear translocation of GAPDH plays an underlying role in the manifestation of disease, such as in, cancer, cardiovascular disease, neurodegeneration, ischemia and major mental illness. Although the oxi-GAPDH stress-signaling cascade is well characterized, whether other molecules can regulate this signaling cascade remains poorly understood. This thesis will focus on the GAPDH-Siah1 stress-signaling cascade and identifies a novel regulator. Chapter I provides an overview of the multifunctional roles of GAPDH within distinct subcellular domains, its medical implications and future perspectives. Chapter II identifies both ASK1 as regulator of the GAPDH-Siah1 stress-signaling cascade and Siah1 as a novel substrate of ASK1. We show that phosphorylation of Siah1 by ASK1 induces nuclear translocation of GAPDH and p300 acetylation. In Chapter III, we conclude that ASK1 and Siah1 might be potential therapeutic targets to regulate the

GAPDH-Siah1 stress-signaling cascade in disease.

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Chapter I: The diverse functions of GAPDH: views from different subcellular compartments

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Abstract

Multiple roles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been recently appreciated. In addition to the cytoplasm where the majority of GAPDH is located under basal conditions, GAPDH is also found in particulate fractions, such as the nucleus, the mitochondria, and small vesicular fractions. When cells are exposed to various stressors, dynamic subcellular re-distribution of GAPDH occurs. Here we review these multifunctional properties of GAPDH, especially linking them to its oligomerization, posttranslational modification, and subcellular localization. This includes mechanistic descriptions of how *S*-nitrosylation of GAPDH under oxidative stress may lead to cell death/dysfunction via nuclear translocation of GAPDH, which is counteracted by a cytosolic GOSPEL. GAPDH is also involved in various diseases, especially neurodegenerative disorders and cancers. Therapeutic strategies to these conditions based on the molecular understanding of GAPDH are discussed.

Introduction

In the past two decades, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was once considered a simple "housekeeping" protein has been shown to be involved in many cellular processes in addition to glycolysis. These include: DNA repair (1), tRNA export (2), membrane fusion and transport (3,4), cytoskeletal dynamics (5), and cell death (6-12). The multifunctional properties of GAPDH are likely to be regulated, at least in part, by its oligomerization, posttranslational modification, and subcellular localization. Posttranslational modifications are divided into reversible and irreversible ones. Here we review this multifunctional nature of GAPDH exhibited in distinct subcellular domains of the cytoplasm, vesicles, mitochondria, and nucleus. We propose a novel concept of how these functions may have a common biological significance in the role of stress response.

1. GAPDH in the cytoplasm

In the cytoplasm GAPDH exists primarily as a tetrameric isoform composed of four identical 37 kDa subunits, each with a single catalytic thiol group. GAPDH coverts glyceraldehyde-3-phosphate to D-glycerate 1,3-bisphospate, in the presence of nicotinamide adenine dinucleotide (NAD^+) and inorganic phosphate, and mediates formation of NADH and adenosine triphosphate (ATP). While GAPDH continues to retain its fundamental role as a glycolytic "housekeeping" protein of the cytoplasm, accumulating evidence indicates that posttranslational modifications of cytosolic GAPDH regulate it's glycolytic activity or push this molecule into functional avenues that deviate from glycolysis.

Protein acetylation, a reversible posttranslational modification involved in regulation of many cellular processes, has been reported to regulate cytosolic GAPDH glycolytic activity (13). In the presence of high glucose levels, acetylation of GAPDH at lysine 254 (K254) via acetyltransferase p300/(CREB-binding protein)-associated factor (PCAF) augments GAPDH enzymatic activity. In contrast, low glucose levels repress GAPDH glycolytic activity by histone deacetylase 5 (HDAC5) mediated deacetylation of the K254 residue on GAPDH (13). This study suggests that regulation of GAPDH activity by PCAF and HDAC5 plays an important role in cancer cells that rely on aerobic glycolysis to coordinate cellular process required for cell proliferation and tumor growth.

In human monocytes GAPDH facilitates a metabolic shift from anaerobic respiration to the pentose phosphate pathway (14). Oxidative stress following the respiratory burst during phagocytosis and monocyte activation induces *S*-thiolation of the reactive sulfhydryl groups on GAPDH. The corresponding cysteine residue of GAPDH

in other organisms is also modified under oxidative stress, which was proposed to be a mechanism to protect the glycolytic enzyme from irreversible oxidative inactivation (15,16). Since inactivation of GAPDH by *S*-thiolation is readily reversible, this posttranslational modification may allow GAPDH to function as a switch that enables cells to shift between metabolic functions and maintenance of oxidation/reduction balance. Indeed, Krobitsch and colleagues (17) provided the first direct evidence that oxidative inhibition of glycolytic enzymes, including GAPDH, is a controlled response that enables cells to redirect their carbohydrate flux from glycolysis to the pentose phosphate pathway, generating NADPH, the reducing power within cells to protect them from oxidative stress (**Fig. 1**).

Other studies have shown that with the redox-sensitive cysteine residue 150, GAPDH can modulate cellular signaling pathways in response to oxidative stress (18,19). For example, GAPDH was shown to physiologically bind to inositol 1,4,5-trisphosphate receptor, this association allows GAPDH to deliver NADH in close proximity to the channel and thus regulates intracellular Ca^{2+} signaling (**Fig. 1**) (20).

S-Nitrosylation, a covalent addition of a nitric oxide (NO) group to the thiol side chain of cysteine, has emerged as an important mechanism for reversible posttranslational regulation of many proteins including GAPDH (21). However, its effect in mediating a metabolic flux is limited due to subsequent posttranslational modifications that inactivate GAPDH irreversibly. These include NAD^+ or NADH attachment, both of which are capable of more strongly inhibiting the catalytic activity of GAPDH (22,23). When the cell is exposed to massive stressors beyond its stress tolerance level, the inactivation of GAPDH may have catastrophic "loss of function" effects by reducing the ability of the

cell to meet the increased energy demands required to maintain homeostasis under extreme stress (24,25). However, posttranslational or conformational modifications to a small pool of the total cellular GAPDH are potentially unlikely to induce dramatic changes in the cellular glycolytic pathways. For example, when a small pool of *S*-nitrosylated GAPDH is further and irreversibly sulphonated (**Fig. 1**), this modification facilitates the translocation of GAPDH to subcellular domains where it does not normally occur and does not significantly impact the metabolic state of the cell. As seen with GAPDH-seven in absentia homolog 1 (Siah1) association, nuclear translocation of GAPDH may stimulate a "gain of function" that could provoke apoptosis or cellular dysfunction (see in subsection 5 below) (6).

The posttranslational modifications from *S*-nitrosylation to sulphonation commit GAPDH to an irreversible signaling cascade that begins in the cytosol and traverses to other cellular compartments. Thus, regulatory mechanisms for this cascade are important for cellular homeostasis. We have recently reported a novel protein, GOSPEL (GAPDH's competitor Of Siah Protein Enhances Life), as a key regulator for GAPDH (26). This cytosolic protein is highly expressed in organs with high levels of energy requirement and high expression levels of GAPDH, such as muscle, heart, and brain. In the presence of nitrosative stress, GOSPEL is quickly S-nitrosylated and retains GAPDH in the cytoplasm, promoting GAPDH-GOSPEL association while competitively preventing the cytotoxic interaction of GAPDH with Siah1 (**Fig. 1**) (26). The competition between GOSPEL and Siah1 for GAPDH binding is likely to maintain cellular homeostasis when cells are exposed to stressors, by favoring the cytoprotective GOSPEL *S*-nitrosylation over the cytotoxic mechanisms mediated by GAPDH *S*-

nitrosylation. However, once the level of nitrosative stress exceeds a threshold, GAPDH-Siah1 binding predominates over GAPDH-GOSPEL interaction and then leads to cell death/dysfunction (26). This is analogous to *S*-nitrosylation of the NMDA-type glutamate receptor (27-29): activation of NMDA receptor at a modest level contains a protective mechanism by *S*-nitrosylation (a type of negative feedback), inhibiting the overactivation of this receptor that might result in massive activation of nNOS (nitrosative stress) and cell death/dysfunction. Likewise, we reported that overexpression of GOSPEL is neuroprotective, whereas mutant GOSPEL lacking the *S*-nitrosylation site and its binding of GAPDH fails to block cell death in primary neuron cultures (26). This neuroprotective action of GOSPEL was further validated in a model of NMDA excitotoxicity *in vivo* (26).

Furthermore, increased levels of oxidative stresses can promote GAPDH aggregation in the cytoplasm, which seems to be associated with cell death (**Fig. 1**) (30,31). Oxidative stress *in vitro* elicits the formation of disulfide-bonded GAPDH aggregates, which in turn results in the production of amyloid-like fibrils (30). Similarly, oxidative stress caused *in vivo* by methamphetamine, which produces massive oxidative stress, induces the formation GAPDH aggregates in mouse brain. In transgenic mice overexpressing wild-type GAPDH methamphetamine accelerated GAPDH aggregation and neuronal cell death (31).

2. GAPDH in association with microtubules, vesicular trafficking, and the cytoskeleton (membrane fusion)

GAPDH was one of the first glycolytic enzymes known to interact with tubulin and actin to facilitate microtubule bundling and actin polymerization, respectively (**Fig. 2**) (5,32). Serum deprivation, likely to be associated with oxidative stress, promotes the association of GAPDH with the stress fibers (microfilament bundles) in NIH 3T3 cells (33). GAPDH-microtubule associations directly modulate the glycolytic activity and quaternary structure of GAPDH by promoting the reversible dissociation of its tetrameric isoform into glycolytically inactive monomeric molecules of GAPDH (34). Furthermore, catalytically active GAPDH may be transported within the cell via microtubule treadmilling during a process that may allow it to couple signal-stimulated glycolysis with the reorganization of the cytoskeleton (**Fig. 2**) (5,32).

Multiple studies from different groups have demonstrated the participation of one or more GAPDH isoforms in membrane fusion and trafficking in biological systems. For example, Robbins and colleagues (35) demonstrated that a mutation of GAPDH altered membrane trafficking in Chinese hamster ovary cells. The small GTPase Rab2 localizes to vesicular tubular clusters (VTCs), which function as transport complexes carrying cargo between the endoplasmic reticulum (ER) and the Golgi complex. Tyrosine phosphorylation of GAPDH by atypical protein kinase Cι (aPKC) is facilitated by Rab 2, which increases phospho-GAPDH recruitment to VTCs. This process plays an important role for membrane trafficking between the ER and Golgi complex and for membrane trafficking from VTCs (4,36,37). A tyrosine kinase Src-mediated phosphorylation of aPKC further facilitates protein associations of Rab2-Src-aPKC-GAPDH on VTCs (38),

and phospho-GAPDH promotes the interaction of the microtubules and motor proteins with Rab2-generated vesicles (**Fig. 2**). Thus, GAPDH might act as an adaptor or scaffolding protein that mediates vesicular trafficking between cellular compartments (**Fig. 2**) (39). Similarly, GAPDH increases the interaction of the microtubules with N-myrisotylated p22, an EF-hand Ca^{2+}-binding protein, which facilitates microtubule-membrane interactions (40). Moreover, enzymatically active GAPDH has been demonstrated to be necessary for fast axonal transport. Scaffolding of GAPDH to vesicles via Huntingtin allows vesicular GAPDH the capacity to provide ATP within close proximity of transport vesicles (41). The ATP generated by GAPDH fuels the molecular motors for fast axonal transport over long distances, in a mitochondrial independent manner.

GAPDH isoforms and/or posttranslational modifications of GAPDH have been demonstrated to play a critical role in membrane fusion. Resolution of GAPDH isoforms by GTP-affinity and Mono Q chromatography has demonstrated that tetramers of glycolytically inactive isoforms of GAPDH catalyze fusion of plasmenylethanolamine containing vesicles (3). GAPDH catalyzed membrane fusion was inhibited by monoclonal antibodies against GAPDH, but not by koningic acid, a known inhibitor of GAPDH glycolytic activity. *In vitro* studies on the rate of fusion catalyzed by glycolytically inactive isoforms of GAPDH suggest that GAPDH is capable of driving fusion of synaptic vesicles with the presynaptic membrane at rates observed *in vivo* (3). This isoform of GAPDH was not fully described in the studies mentioned; therefore one or several posttranslational modifications of GAPDH could be involved in regulating membrane fusion. In deed, tubulin modulates GAPDH-catalyzed membrane fusion and

phosphorylation of GAPDH via aPKC has been reported to counteract tubulin-mediated inhibition of GAPDH-catalyzed membrane fusion (36,42). Recent studies have demonstrated that posttranslational deamidation of GAPDH by transglutaminase 2 increased localization of GAPDH to the plasma membrane and regulated membrane fusion (43). Glutamine to glutamate mutations of one to seven of the glutamines present on GAPDH suggests that posttranslational deamidation of multiple but not specific glutamines might be to sufficient to regulate membrane fusion. These studies suggest that the functional roles of GAPDH in cytoskeletal reorganization, membrane fusion and vesicular trafficking are tightly regulated via posttranslational modifications to small pools of GAPDH that are restricted to very limited subcellular locations.

3. GAPDH in the mitochondria

At basal conditions the levels of GAPDH in the mitochondria are relatively low. However, mitochondrial GAPDH levels are rapidly elevated when cells are exposed to serum deprivation and DNA-damaging agents (44). Previous studies have also demonstrated that rotenone, a common mitochondrial complex I inhibitor, induces enrichment of cytosolic GAPDH to nuclear and mitochondrial fractions (45). Analysis of GAPDH within these subcellular fractions revealed disulfide-bonded GAPDH dimers or oligomers.

When GAPDH is expressed exogenously, a pool of GAPDH is translocated to the mitochondria where it induces pro-apoptotic mitochondrial membrane permeabilization (MMP) via an association with the voltage-dependent anion channel 1 (VDAC1) (44). Studies with isolated mitochondria suggest that dimers and tetramers of GAPDH interact with VDAC1. Exogenous expression of GAPDH in the mitochondria cause loss of the inner transmembrane potential, matrix swelling, permeabilization of the inner-mitochondrial membrane, and the release of two pro-apoptotic proteins, cytochrome c and apoptosis-inducing factor (**Fig. 3**) (44).

In contrast, another study reports that GAPDH participates in recovery from mitochondrial outer-membrane permeabilization (MOMP) (46). In this scenario, GAPDH protects cells from death following MOMP, in the absence of caspase activation (**Fig. 3**). Here GAPDH may provide enough ATP to maintain the mitochondrial membrane potential via the F_0F_1 ATPase, helping to counteract the effects of the energetic collapse, thereby promoting cell survival. Alternatively, stabilization of activated Akt by overexpression of GAPDH indicates that GAPDH sustains Akt activity

and leads to phosphorylation of FoxO, Bcl-6 downregulation and expression of Bcl-xL (47). Expression of Bcl-xL allows recovery of some mitochondria from MOMP and promotes cell survival.

Protection from mitochondria-mediated cell death is also accomplished by elimination of damaged mitochondria through mitophagy. Cardiomyocytes exposed to ischemia and reperfusion or reoxygenation (I/R) injury exhibit translocation of GAPDH in association with mitophagy. In a cell culture model and whole hearts, I/R was shown to induce translocation of a catalytically inactive GAPDH isoform to the mitochondria (48). I/R induced translocation of GAPDH to the mitochondria is dependent on the absence of phosphorylation at threonine residue 246 on GAPDH (48). Inhibition of protein kinase C δ (PKCδ) demonstrated that a GAPDH/ PKCδ signaling switch regulates GAPDH-driven mitochondrial elimination via phosphorylation at T246 (48).

Together, these studies demonstrate that GAPDH translocates to and regulates cellular processes in association with the mitochondria. However, for most cases it remains unclear which specific posttranslational modifications play a role in regulating translocation of GAPDH to the mitochondria or to specific mitochondrial proteins.

4. GAPDH in the nucleus

We reported that a small pool of GAPDH is translocated to the nucleus upon exposure to stressors and participates in cell death/dysfunction (12) with other groups also replicating this observation (10,49-56). This indicates that GAPDH may act as a relay molecule between cellular compartments during cellular stress. The signal is conveyed by GAPDH that is S-nitrosylated by NO at active site Cys-150, allowing GAPDH to bind to Siah1 (an E3 ubiquitin ligase), leading to nuclear translocation of the GAPDH-Siah1 complex (**Fig. 4**) (6). It seems that the nuclear localization signal on Siah1 drives translocation of the GAPDH-Siah1 protein complex, probably while maintaining the tetrameric structure of GAPDH. Stabilized Siah1 together with *S*-nitrosylated GAPDH facilitates ubiquitination and degradation of the nuclear co-repressor N-CoR (6,57). Further studies have also shown that nuclear translocated GAPDH is further acetylated at Lys-160 by the histone acetyltransferase p300/CBP via direct protein interaction, which in turn stimulates the catalytic activity of p300/CBP. This nuclear event leads to the acetylation of downstream targets, including the tumor suppressor p53 (**Fig. 4**) (58). In the nucleus, nitrosylated GAPDH transfers its nitric oxide to sirtuin-1 (SIRT1), histone deacetylase-2 (HDAC2) and DNA-activated protein kinase (DNA-PK), thereby regulating the activity of nuclear proteins via transnitrosylation (59). Through these mechanisms, the nuclear GAPDH-Siah1 complex may regulate gene expression via degradation of transcriptional coregulatory proteins and by modulating histone modifications, which may result in cell death, cellular dysfunction or stress response.

GAPDH was shown to co-immunoprecipitate with promyelocytic leukemia protein (PML) and co-localize in a subset of nuclear bodies (**Fig. 4**) (60). The localization of PML and GAPDH to the same nuclear bodies is reportedly dependent on the presence of RNA (60). Since disruption of PML bodies reduces apoptosis in acute promyelocytic leukemia and GAPDH induces apoptotic neuronal death, the GAPDH-PML interaction may be involved in the regulation of cell death. During hyperglycemia-induced oxidative stress, poly (ADP-ribose) polymerase -1 (PARP-1), a DNA-repair enzyme that is activated by severe DNA damage, regulates GAPDH activity by poly(ADP-ribosyl)ation (**Fig. 4**) (61). Inactivation of GAPDH via ADP-ribosylation elicits greater cellular energy deficits and accelerates cell death. Through this process, GAPDH is believed to not only relay stress signals but also shuttle the generation of ATP to cells responsible for repairing and/or removing dead or dying cells, such as those surrounding ischemic tissue (62).

Nuclear GAPDH also has various functions unrelated to cell death. The correlation of increased uracil DNA glycosylase (UDG) activity with an increase in cell cycle-regulated expression of monomeric GAPDH suggests that this multifunctional molecule also plays an important role in DNA repair (**Fig. 4**) (63,64). The cell-cycle dependent expression of GAPDH in this study suggests that GAPDH has selective functions at specific phases in the cell cycle. GAPDH directly interacts with SET nuclear oncogene, a molecule that inhibits cyclinB-cdk1 activity. GAPDH-SET interaction reverses the inhibitory effects of SET leading to an advanced cyclin B-cdk1 activity peak, increased mitosis, and acceleration of the cell cycle (**Fig. 4**) (65). In HeLa cells, monomeric nuclear GAPDH associates with DNA as a component of the multicomplex

Oct-1 coactivator (OCA-S) and stimulates expression of Histone 2B during S-phase (66). Monomeric nuclear GAPDH acts as a redox sensor that regulates Histone 2B transcription in a NAD^+/NADH redox-dependent manner (**Fig. 4**) (67). GAPDH-NAD^+ association is required for Oct-1-mediated gene transcription. *O*-linked N-acetylglucosamine modifications of GAPDH (*O*-GlcNAcylation mainly on Thr227) are able to disrupt the tetrameric form of GAPDH, enabling its nuclear translocation (68). Thus, it may be important to explore whether *O*-linked N-acetylglucosamine modifications may underlie roles of monomeric nuclear GAPDH.

GAPDH can also physically interact with apurinic/apyrimidinic endonuclease (APE1), an essential enzyme that functions in the base excision DNA repair pathway to process spontaneous and drug-induced abasic or apurinic/apyrimidinic sites, as well as, to regulate the redox state of a number of transcriptional factors (**Fig. 4**) (69). GAPDH reactivates the endonuclease activity of APE1 by converting the oxidized APE1 to the reduced form, restoring its ability to cleave abasic sites. Nuclear GAPDH also plays a role in maintaining and protecting telomeric DNA from rapid degradation (**Fig. 4**) (70,71).

5. Significance of multifunctional roles for GAPDH

Several distinct pools of GAPDH appear to sense intra- and extracellular stressors via posttranslational and/or conformational changes and activate downstream pathways to maintain homeostasis or promote cell death (summarized in **Table. 1**). The prominent expression of GAPDH in the cytosol may allow it to function efficiently as an intracellular sensor capable of directly relaying signals to various organelles, such as the nucleus. GAPDH functions as a double-edged sword capable of facilitating the completion of an apoptotic event or regulating the recovery from an insult, such as that seen following MOMP in the mitochondria. Of note, apoptotic cell death can contribute to the maintenance of homeostasis at the organism level. At the cellular level the multifunctional roles of GAPDH maintain homeostasis within subcellular niches. Glycolytic enzymes function in a well-coordinated manner, and some of them, such as GAPDH and aldolase, form protein-protein complexes (72). In analogy to GAPDH, other glycolytic enzymes may have multiple roles and function as intracellular sensors. The relationship of GAPDH with other glycolytic enzymes and their roles beyond glycolysis may be an interesting subject to be studied in the future. GAPDH is evolutionally well preserved. How did GAPDH acquire such a wide range of cellular roles during the evolution? This may be an interesting question to address in regard to homeostatic regulation in organisms.

6. Medical implications and future perspectives

Participation of GAPDH in multiple pathways of homeostatic regulation indicates that this molecule may also play a role, when it is disturbed, in the manifestation of certain diseases. Accumulating evidence suggests that nuclear GAPDH may be involved in several neurodegenerative disorders (73). Nuclear GAPDH has been found in fibroblasts and in postmortem brains from patients with polyglutamine diseases (such as Huntington's disease or dentatorubral-pallidoluysian atrophy) (74,75), Parkinson's disease (56), and Alzheimer's disease (53,76). Some studies suggest that GAPDH interacts with β-amyloid peptides, mutant huntingtin, the androgen receptor, and atrophin-1 (77-81). Functional analyses have demonstrated that GAPDH negatively affects the stability of *SCN1A* mRNA encoding the voltage-gated sodium channel α-I subunit involved in Dravet syndrome (82). In an experimental model of brain ischemia, accumulation of nuclear GAPDH is observed (55). More recently, a mouse model for neuropsychiatric illness expressing a putative dominant-negative disruption in schizophrenia 1 (DN-DISC1) was shown to have augmented GAPDH-Siah1 binding in the frontal cortex (83). Augmentation of this oxidative stress-associated cascade in this mouse model suggests that the GAPDH-Siah1 pathway may also underlie cognitive and motivational impairments observed in major mental illnesses.

Moreover, promising pharmacological evidence supports the notion that nuclear GAPDH plays an important role in cell dysfunction and death. Deprenyl, a classic monoamine oxidase B (MAO-B) inhibitor, used for symptomatic amelioration in patients with Parkinson's disease also blocks GAPDH-Siah1 binding and reduces nuclear translocation of GAPDH and cell death (84-87). Rasagiline, another MAO-B inhibitor

was also shown to have neuroprotective effects in ethanol-induced cell death mediated by a novel GAPDH-MAO-B pathway (88,89). Some structural derivatives of deprenyl, even those lacking the inhibitory action on MAO-B, are also neuroprotective (84-87,90-92). Among them, TCH346 shows neuroprotective action largely via blockade of GAPDH-Siah1 binding and nuclear translocation of the GAPDH-Siah1 protein complex (93). To the contrary, saframycin, an antiproliferative agent for the treatment of leukemia- and tumor-derive cells is capable of forming a ternary complex with GAPDH and DNA to induce cytotoxic effects (94). Further understanding of GAPDH may aid in the development of novel therapeutic strategies for many disorders.

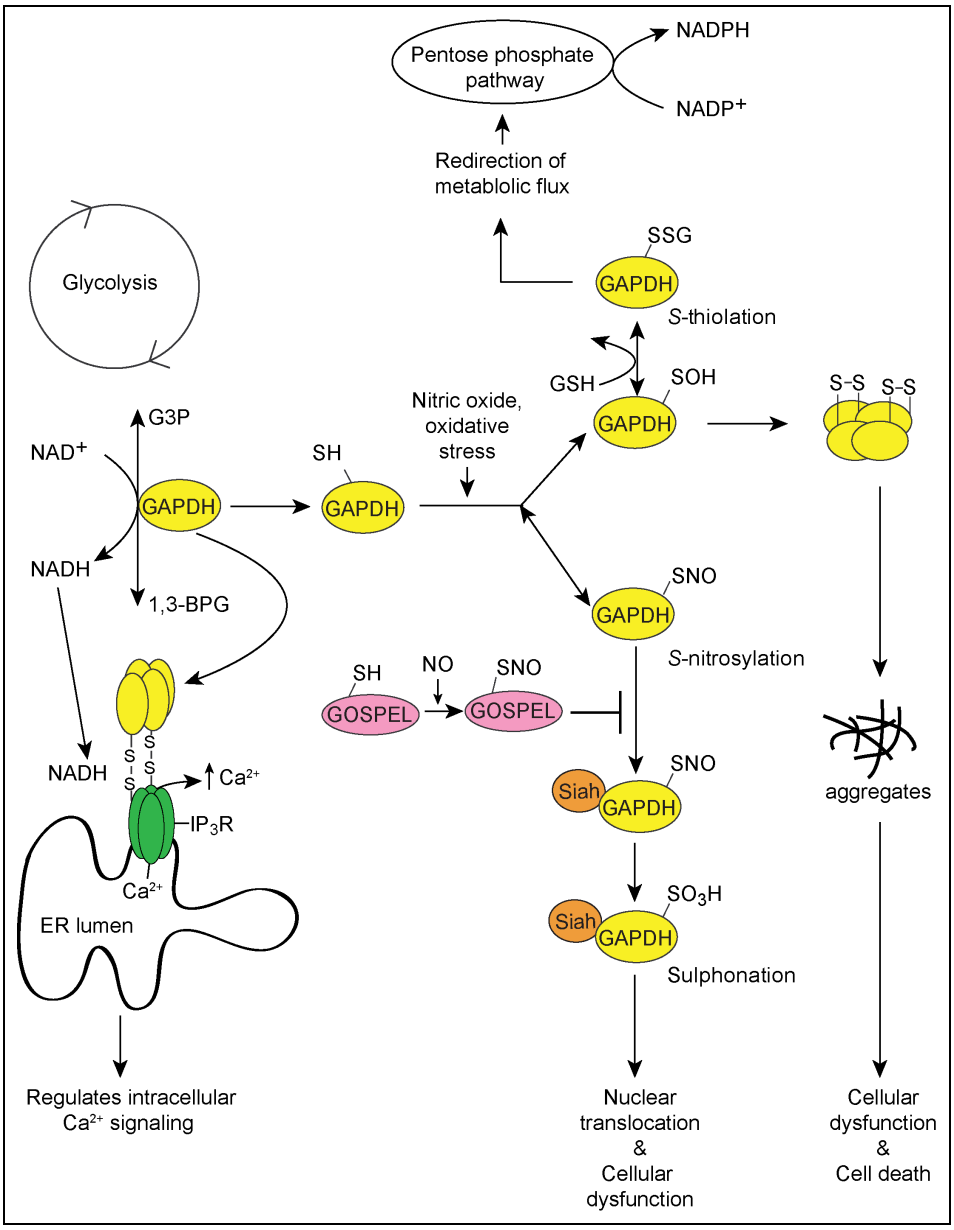

Fig. 1. GAPDH in the cytoplasm: Glycolytic enzyme, GAPDH, catalyzes the conversion of glyceraldehyde-3-phosphate (G3P) into 1,3-biphosphoglycerate (1,3-BPG). GAPDH can undergo different posttranslational modifications, which may determine some of its non-glycolytic functions. Under conditions of cellular oxidative stress, GAPDH can undergo reversible *S*-thiolation (-SSG), a mechanism to protect the glycolytic enzyme from irreversible oxidative inactivation and consequently redirecting the metabolic flux from glycolysis to the pentose phosphate pathway to maintain an optimal NADPH/$NADP^+$ ratio. GAPDH contributes to local $NADH^+$ and may regulate IP_3R-mediated Ca^{2+} signaling. Nitric oxide stress also leads to reversible *S*-nitrosylation (-SNO) of cystine-150 of GAPDH that facilitates its binding to Siah1, and results in translocation of the complex to the nucleus and irreversible sulphonation (-SO_3H) of GAPDH. This cascade mediates cell death/dysfunction in a gain-of-toxic manner. *S*-Nitrosylation of GOSPEL augments binding of GOSPEL with GAPDH, competing with binding of Siah1 with GAPDH, which is a cytoprotective mechanism against GAPDH-Siah1 cascade. Exposure to oxidants can induce an irreversible oxidation of cysteine residues that favor intermolecular disulfide bonds and the subsequent formation of cytosolic aggregates of GAPDH. This insoluble protein may ultimately promote cellular dysfunction and cell death.

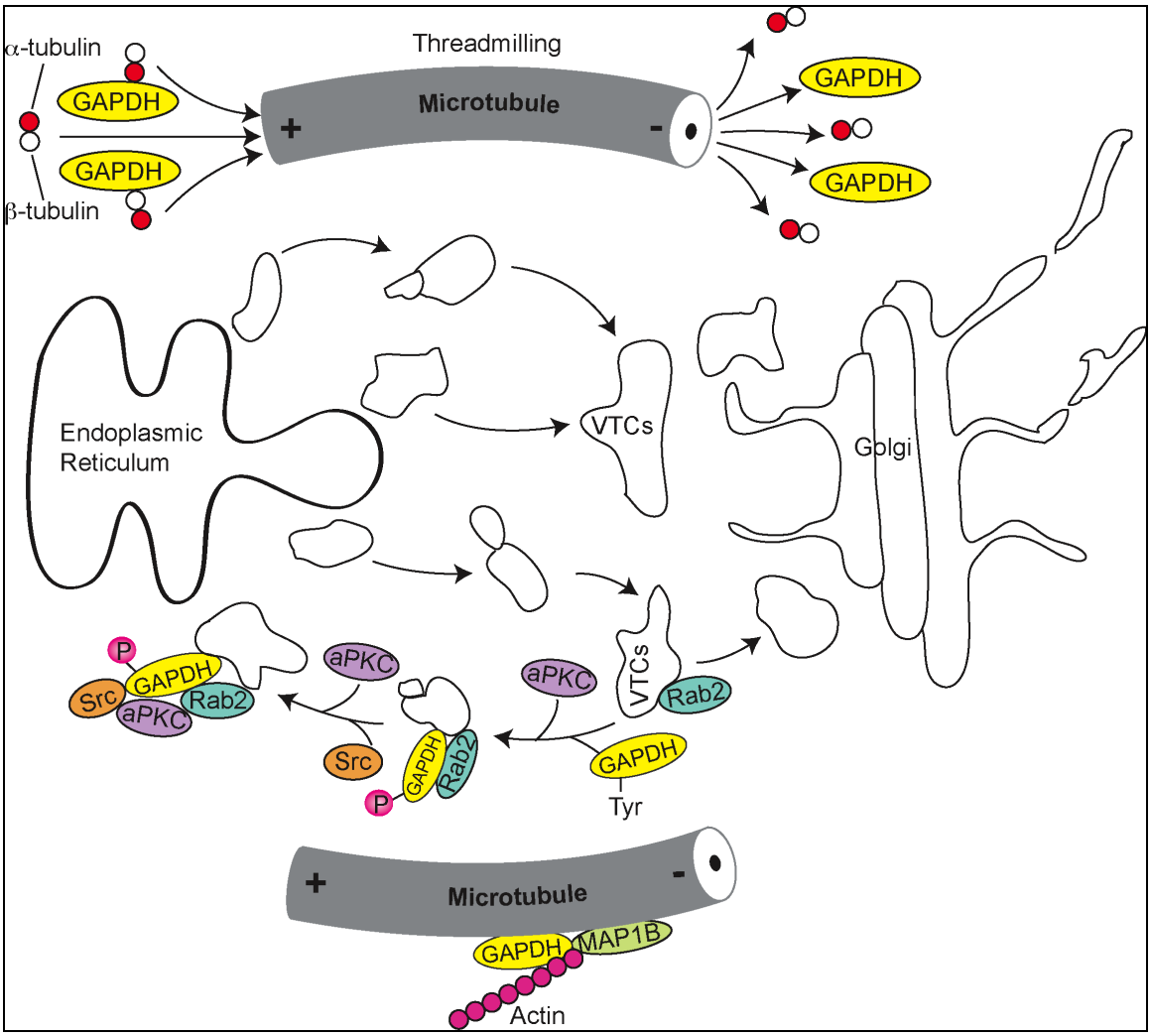

Fig. 2. GAPDH in association with the cytoskeleton and vesicular transport: GAPDH is known to interact with tubulin and actin, facilitating microtubule bundling and actin polymerization, respectively. GAPDH may be transported within the cell via microtubule treadmilling. GAPDH also plays an important role in VTCs (vesicular tubular clusters) where phosphorylated GAPDH by atypical protein kinase Cι (aPKC) is recruited. This process plays an important role for membrane trafficking between the ER and Golgi complex without requirement of GAPDH glycolytic activity. A tyrosine kinase Src-mediated phosphorylation of aPKC further facilitates protein associations of Rab2-Src-aPKC-GAPDH on VTCs, and the phospho-GAPDH promotes the interaction of the microtubules and motor proteins with Rab2-generated vesicles.

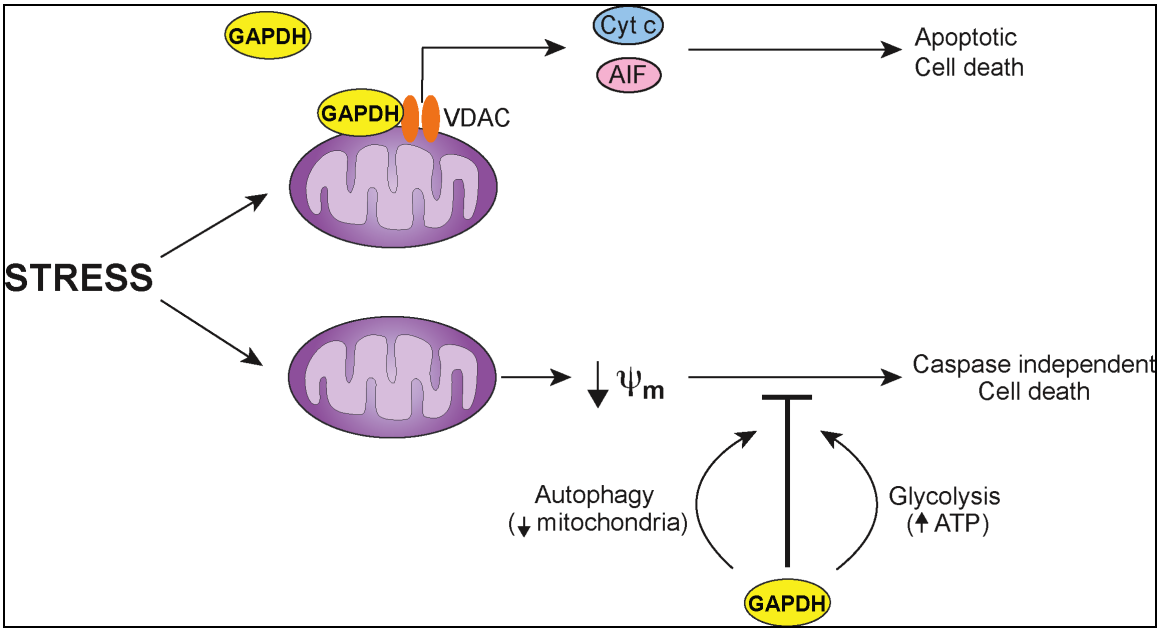

Fig. 3. GAPDH in the mitochondria: GAPDH localized in the mitochondria has distinct functions in a context-dependent manner. GAPDH can bind with the voltage-dependent anion channel (VDAC), which may promote the release of cytochrome c (CytC) and apoptosis-inducing factor (AIF), leading to apoptotic cell death. In stressed conditions, a decrease in mitochondrial membrane potential (ψ_m) leads to caspase-independent cell death (CICD). In this context, GAPDH can inhibit cell death by simultaneously increasing ATP levels through glycolysis and stimulating autophagy-mediated clearance of permeabilized mitochondria.

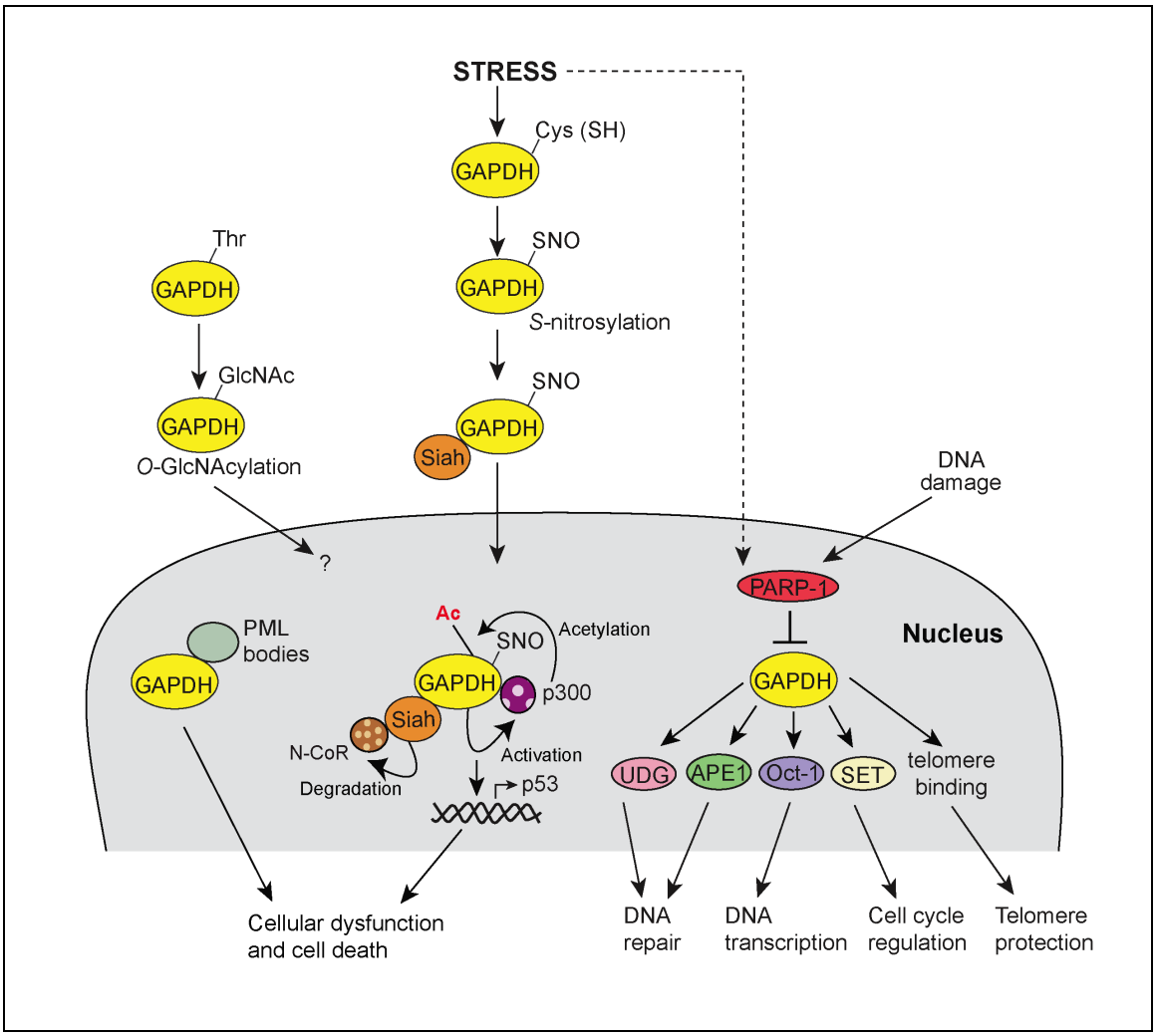

Fig. 4. GAPDH in the nucleus: Oxidative modifications such as, *S*-nitrosylation of GAPDH, increase binding to Siah1, which mediates its nuclear translocation. GAPDH stabilizes Siah1 and facilitates Siah1-mediated degradation of nuclear proteins such as, nuclear co-repressor (N-CoR). Nuclear GAPDH is acetylated by the p300/CREB-binding protein (CBP), which in turn stimulates the catalytic activity of p300/CBP. Consequently, downstream targets of p300/CBP, such as p53, can be activated and cause cellular dysfunction. In addition, GAPDH can undergo O-linked beta-N-acetylglucosamine glycosylation (O-GlcNAcylation) at threonine residues, which also mediates its nuclear translocation. Nuclear GAPDH binding with PML is associated with cell death. Nuclear GAPDH can participate in DNA repair, regulations of gene transcription and cell cycle and turnover of telomeric DNA. During oxidative stress, PARP-1 may serve as an upstream regulator of nuclear GAPDH.

Modification of GAPDH	Amino Acid Site	Structural Form	Reversible	Catalytic Activity	Functional Relevance
Acetylation	K160 K256	Tetramer	Yes	Yes/No	Stress Response Cytotoxicity Cellular Dysfunction Cell Proliferation
O-GlcNacylation	T227	Monomer	Yes	No	Stress Response Cytotoxicity Cellular Dysfunction Cell Proliferation
Phosphorylation	Y40 A81 S149 T182 T244	Unknown	Yes	Unknown	Stress Response Cytotoxicity Cellular Dysfunction Cellular Trafficking
S-nitrosylation	C150	Tetramer Monomer Dimer	Yes	No	Stress Response Cytotoxicity Cellular Dysfunction
S-thiolation	-SH groups	Tetramer Monomer Dimer	Yes	No	Stress Response Cytotoxicity Cellular Dysfunction
Sulphonation	C150	Tetramer Monomer Dimer	Yes	No	Stress Response Cytotoxicity Cellular Dysfunction
Aggregation	C150 and C282	Aggregate	No	No	Stress Response Cytotoxicity Cellular Dysfunction
Gospel attachment	P80 - S120	Monomer?	Yes?	No	Stress Response Cytotoxicity Cellular Dysfunction
NAD/NADH attachment	Rossmann-fold	Tetramer	No	No	Stress Response Cytotoxicity Cellular Dysfunction
Covalent NADH attachment	C150	Tetramer	No	No	Stress Response Cytotoxicity Cellular Dysfunction
Polyglutamine association	M1 – V169	Tetramer Monomer Dimer	No	Yes/No	Stress Response Cytotoxicity Cellular Dysfunction
Siah attachment	P220 - (K225*) - V238	Tetramer	No	No	Stress Response Cytotoxicity Cellular Dysfunction

Table 1. Summary of posttranslational and/or conformational changes of GAPDH.

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Chapter II: Role of Apoptosis Signal-regulating Kinase 1 (ASK1) as an Activator of the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Stress-Signaling Cascade

ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays roles in both energy maintenance and stress-signaling by forming a protein complex with seven in absentia homolog 1 (Siah1). Mechanisms to coordinate its glycolytic and stress cascades are likely to be very important for survival and homeostatic control of any living organism. Here we report that apoptosis signal-regulating kinase 1 (ASK1), a representative stress kinase, forms a ternary complex with GAPDH and Siah1 and is able to phosphorylate Siah1 at specific amino acid residues (T70/T74 and T235/T239). Phosphorylation of Siah1 by ASK1 triggers GAPDH-Siah1 stress-signaling and activates a key downstream target, p300 acetyltransferase, in the nucleus. This novel mechanism, together with the established *S*-nitrosylation/oxidation of GAPDH at C150, provides evidence of how GAPDH stress-signaling is finely regulated. In addition, the present results imply crosstalk between the ASK1 and GAPDH-Siah1 stress cascades.

INTRODUCTION

Survival of living organisms is dependent on homeostatic control of energy maintenance and a flexible response to environmental stressors (1-4). In complex living organisms, which are comprised of multiple cells and organs, survival of each cell and elimination of damaged cells becomes important for homeostatic control (5-8). Despite the importance of this fundamental concept, the molecular machinery underlying these mechanisms and their coordination are not fully understood.

A major mechanism for supplying cellular energy is glycolysis, in which glucose is catabolized to pyruvic acid via several enzymatic reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis that plays a major role in cellular energy supply (9). In addition to this classic role, GAPDH is involved in many subcellular processes that include; DNA repair (10), membrane fusion and transport (11), tRNA export (12) and cell death (13-19). The functional diversity of GAPDH is largely regulated by its subcellular localization and posttranslational modifications (20). Recently studies have revealed that GAPDH can be oxidized and/or *S*-nitrosylated under stress conditions. Following this posttranslational modification GAPDH is then translocated to the nucleus as a complex with Siah1, which has a strong nuclear localization signal (21). In the nucleus, GAPDH modulates several proteins, in particular stimulating the catalytic activity of acetyltransferase p300/CREB binding protein (CBP) that regulates transcription of various genes (22). Therefore, GAPDH may modulate homeostatic control by bridging energy supply (glycolytic pathway) to stress response (GAPDH-Siah1 cascade), which is finely regulated by posttranslational modifications (23-26).

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family. Although cellular substrates of ASK1 have not yet been fully studied, MAPKK4/7 (a kinase that phosphorylates JNK) and MAPKK3/6 (a kinase that phosphorylates p38) are well-established substrates (27). ASK1 is activated in response to oxidative stress, endoplasmic reticulum stress, and other forms of cellular stress (28-30). In addition, ASK1 plays pivotal roles in a wide variety of cellular responses, which include, but are not limited to, apoptosis (31,32). Dysregulation of the ASK1 signaling pathway is closely linked to various diseases, such as cancer, cardiovascular diseases, diabetes, and neurodegenerative diseases including polyglutamine-induced neurodegeneration and Parkinson's disease (29,33-39).

The primary focus of the present study is to elucidate regulatory mechanisms of the GAPDH-Siah1 pathway. Here we report that ASK1 phosphorylates Siah1 and critically modulates the GAPDH-Siah1 pathway via direct protein interactions.

EXPERIMENTAL PROCEDURES

Chemicals, plasmids, and antibodies—All reagents were purchased from Sigma, unless noted otherwise. cDNA constructs for ASK1 were prepared as previously published (40,41). GST-tagged-ASK1, GAPDH and GST only constructs were cloned into the pGEX-4T-1 vector. GST-tagged-Siah1 constructs were cloned into the pGEX-5X-2 vector. HA- and FLAG-tagged-ASK1 constructs were cloned into the pcDNA3 vector. Myc-tagged-Siah1 constructs were cloned into the pRK5 vector. Myc-tagged-Siah1 and its threonine to alanine (T to A) mutants were prepared using the Quick Change site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene). Single mutants with threonine to alanine mutations are: M1, mutant with T7A and T11A; M2, mutant with T70A and T74A; M3, mutant with T108A and T112A; and M4, mutant with T235A and T239A mutations. Double mutants are M1+M3 and M2+M4.

[Siah1 mutant primers]

M1 (T7A and T11A):

Forward, 5'-GCCAGACTGCTGCAGCATTACCCGCCGCCACCTC-3'

M2 (T70A and T74A):

Forward, 5'-CGCCCCAAACTTGCATGTTGTCCCGCCTGCCGGGGC-3'

M3 (T108A and T112A):

Forward, 5'-TGTGAGATAGCTCTGCCGCACGCCGAAAAGGCAG-3'

M4 (T235A and T239A):

Forward, 5'-CGGCGATTGGCTTGGGAAGCCGCCCCTCGGTCT-3'

Recombinant human GAPDH and human ASK1 [amino acids (aa) 649-946] were purchased from Sigma and Cell Sciences, respectively. The p38 inhibitor SB203580 and JNK inhibitor SP600125 were purchased from Alexis Biochemicals. Antibodies for GAPDH (clones V18 and 6C5), Siah1 (P-18), Myc (9E10), p-Thr (H2) and p300 (C-20) were from Santa Cruz Biotechnologies, p-ASK1(T845), p-p38, p-Jun, Ac-p300 (Lys 1499) were from cell signaling, HA (16B12) was from Clontech, GST (4C10) was from Covance and FLAG (M5) from sigma, LDH, ASK1 and nuclear matrix protein p84 (5E10) were from Abcam. HRP- conjugated mouse/rabbit antibodies were from GE Healthcare, and goat HRP was from Santa Cruz. γ-^{32}P-ATP was from Perkin-Elmer.

Expression and purification of recombinant proteins—The pGEXT vectors carrying GST-tagged-GAPDH, ASK1 (aa 1-940), ASK1 (aa 646-946) or WT Siah1 were transformed into *E. coli* strain DH5α, and the proteins were purified as described (42). Briefly, the *E. coli* DH5α were grown at 23°C for 2 h, induce with 0.1 mM IPTG for 4 h or until OD reached 0.6, then cooled on ice water bath for 4 h. Recombinant proteins were purified from *E. coli* with glutathione sepharose beads.

Animals—C57/BL6 adult mice were purchased from Charles River.

Cell culture—HEK293 cells were grown in Dulbecco's modified medium with 10% FBS and 1% pen-strep. Polyfect (Qiagen) or Lipofectamine 2000 (Life Technologies) were used for transfection of HEK293 cells. Cells were harvested 48 h after transfection. To inhibit ASK1 activity, cells were pretreated with 10 μM NQDI for

1 h prior to treatment with H_2O_2. To activate a cellular stress response, cells were treated with 1 mM H_2O_2 as indicated.

Co-immunoprecipitation (co-IP)—Cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1 mg/ml BSA, protease inhibitor cocktail), co-IPed, and Western blotted as previously described (21,22). Briefly, total cell lysates were incubated with primary antibodies overnigth, precipitated with Trueblot beads (Rockland) and immunoprecipitates were analysed with SDS-PAGE, followed by Western blotting. For sequential co-IP studies, the first co-IP reactions were performed with an anti-HA antibody and eluted with HA peptide at 30°C for 30 min. Elutes were subjected to a subsequent co-IP with an anti-Myc antibody. The final co-IP was then subject to Western blot analysis with an anti-FLAG antibody.

Extraction of nuclear and cytoplasmic proteins—Nuclear and cytoplasmic extracts were prepared using the Biovision nuclear/cytosol extraction kit according to the manufacturer's instructions.

In vitro binding assays—ASK1-Siah1 and ASK1-GAPDH *in vitro* binding assays were performed with equal molar concentrations of GST-tagged-ASK1, Siah1 and His-tagged-GAPDH and incubated in binding buffer (0.1% NP40, 0.5 mM DTT, 10% glycerol, 1 mM PMSF and 2 μg/ml aprotinin in PBS) for 2 h at 4°C. To measure the effects of GAPDH on ASK1-Siah1 binding, 0, 1 and 3 (GAPDH:Siah1) molar concentrations of recombinant GAPDH, with respect to Siah1, were incubated in binding

buffer, as mentioned below. To obtain recombinant GAPDH and Siah1 without the GST tag, GSH sephorose bounded protein was released via thrombin digestion, dialyzed in Slide-A-Lyzer dialysis cassettes (Pierce) and protein purity was analyzed by Western blot. All *in vitro* binding assays were done by GST pull-down via incubation with GSH sepharose beads (50% slurry) for 1 h, the samples were centrifuged at 4000 rpm for 1 min, washed three times in binding buffer, and resuspended in LDS sample buffer (Invitrogen) with 5% β-mercaptoethanol (Sigma) and then heated at 95°C for 5 min. Western blot analysis of the protein precipitates were done using anti-GAPDH, Siah1, and GST antibodies.

In vitro kinase assay—In vitro phosphorylation assays were performed by incubation of recombinant Siah1, GAPDH and GST with or without human recombinant ASK1 (aa 649-946) protein (Cell Sciences) in kinase buffer (4 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM EDTA, 4 mM $MgCl_2$, 0.05 mM DTT, 40 ng/μl BSA, PIC1 and 2 (Sigma), and 10 mM γ-^{32}P-ATP) at 30°C for 30min. *In vitro* phosphorylated proteins were subjected to SDS-PAGE and examined by autoradiography at -80°C with intensifying screen.

p38/JNK experiments—HEK293 cells expressing HA-GAPDH, Myc-Siah1, and HA-ASK1 were treated with 20 μM p38 inhibitor (SB203580) or JNK inhibitor (SP600125) for 1 h. Cell lysates were subjected to co-IP followed by Western blot as previously described (21,22).

Statistical analysis—Two-group analysis was performed by *t*-test (paired or unpaired as appropriate). A value of $p < 0.05$ is considered significant. All data was obtained from the results of three or four independent experiments. Optical density of immunoreactivity in Western blotting was obtained using Image J software.

RESULTS

GAPDH and Siah1 bind to ASK1 and form a ternary complex in cells—GAPDH-Siah1 and ASK1 have been reported to independently play roles in several pathological brain conditions, and are commonly shown to be key stress mediators (26,31,43-45). Thus, we hypothesized that Siah1 and GAPDH might interact with ASK1 at the molecular level. To address this question, we examined mouse brain lysates and observed endogenous protein interactions of ASK1-Siah1, ASK1-GAPDH and GAPDH-Siah1 by co-IP (Fig. 1*A*). Since ASK1 and GAPDH are both well-established sensors of oxidative stress we hypothesized that oxidative stress may promoter the formation of a ternary complex among this three molecules. To test whether or not ASK1, Siah1 and GAPDH formed a ternary complex, we performed sequential co-IPs on cell lysates from HEK293 cells expressing ASK1, Siah1 and GAPDH and observed ASK1-Siah1-GAPDH ternary complex formation is induced by treatment with H_2O_2 (Fig. 1*B*, *upper panel*). Previous studies have reported stimulation of ASK1 activity by H_2O_2 (46). To determine if H_2O_2-stimulated ASK1 activity may be associated with ternary complex formation we measured ASK1 activity via phosphorylation of ASK1 at T845 and demonstrated that ASK1 activity correlated with ASK1-Siah1-GAPDH ternary complex formation (Fig. 1*B*, *lower panel*). These results suggest that ASK1, Siah1 and GAPDH form a ternary complex in response to extracellular stressors.

Direct ASK1-Siah1 binding and modulation by GAPDH in vitro—GAPDH and Siah1 are known to bind directly (21). To characterize the interaction of Siah1 and GAPDH with ASK1 we purified recombinant proteins for *in vitro* binding studies.

Incubation of recombinant Siah1 together with glutathione *S*-transferase (GST) or GST-tagged-ASK1 (aa 1-940) demonstrated that Siah1 binds directly to the N-terminal region of ASK1 (Fig. 2*A*). In contrast, incubation of recombinant GAPDH together with GST or GST-tagged-ASK1 indicated that GAPDH does not directly to ASK1 *in vitro* at least under this condition (data not shown). To test whether GAPDH may modulate ASK1-Siah1 binding we performed *in vitro* binding assays with recombinant Siah1 and ASK1 (aa 1-940) in the presence of increasing amounts of GAPDH. These studies demonstrated that a three molar equivalent of GAPDH augmented ASK1-Siah1 direct binding (Fig. 2*B*). ASK1 (aa 1-940) contains the ASK1 kinase domain (aa 646-946) (27). To determine if Siah1 binds within the kinase domain we conducted *in vitro* binding studies with recombinant Siah1 and fragments of the ASK1 kinase domain and demonstrated that Siah1 binds to ASK1 fragment aa 646-846 of the kinase domain three times more when compared to that of aa 646-946 (Fig. 2*C*). Comparison of Siah1 binding with ASK1 fragment aa 646-946 and aa 746-946 indicates that the aa 696-746 region is critical for binding with Siah1 (Fig. 2*C*). In contrast, Siah1 binding to ASK1 fragment aa 646-846 compared to the fragment aa 646-896 indicates that the aa 846-896 region may have an inhibitory effect (Fig. 2*C*). The notion of these positive and inhibitory domains can account for the overall results.

ASK1 phosphorylates Siah1 in cells and in vitro—Given that Siah1 directly binds within the kinase domain of ASK1, we hypothesized that Siah1 might be a novel substrate of ASK1 phosphorylation. To investigate whether ASK1 could phosphorylate Siah1 in cells, we stimulated ASK1 activity by treating HEK293 cells expressing ASK1

and Siah1 with H_2O_2 and observed augmented Siah1 phosphorylation in an ASK1-activity dependent manner (Fig. 3*A*). Based on these results we hypothesized that ASK1 may directly phosphorylate Siah1, to address this question we conducted phosphorylation studies using recombinant proteins *in vitro*. Phosphorylation of GST-tagged Siah1, but not GST, was observed when Siah1 was incubated with the constitutively active kinase domain of ASK1 (aa 646-946) (Fig. 3*B*). To assertain that phosphorylation of Siah1 was indeed mediated by ASK1 we conducted *in vitro* phosphorylation studies in the presence of NQDI-1 (an specific inhibitor of ASK1) and observed that ASK1 inhibition reduced Siah1 phosphorylation (Fig. 3*C*).

Since we had shown that GAPDH facilitated ASK1-Siah1 binding, we hypothesized that GAPDH may also augment phosphorylation of Siah1. Therefore, we conducted additional *in vitro* phosphorylation studies in the presence of increasing amounts of GAPDH. These studies revealed that in the presence of the kinase domain of ASK1 (aa 646-946) GAPDH did not modulate phosphorylation of Siah1 (Fig. 3*D*).

ASK1 phosphorylates Siah1 at specific phosphorylation motifs—Since ASK1 has been reported to phosphorylate substrates carrying the (S/TxxxS/T) consensus motif (47,48), we examined the amino acid sequence of Siah1 and identified four potential phosphorylation sites which we designated M1, M2, M3, and M4, respectively (Fig. 4*A*). To characterize the ASK1 phosphorylation sites on Siah1, we generated mutants of Siah1 with point mutations (threonine to alanine substitutions) at each consensus sequence and examined how these mutations affected phosphorylation of Siah1 by ASK1 via *in vitro* kinase assays (Fig. 4*B*). Amino acid substitution at M2 led to significant decreases in the

phosphorylation of Siah1 by ASK1. However, Siah1 phosphorylation by ASK1 was reduced the most when we introduced mutations at both M2 and M4 (M2+M4) (Fig. 4*B*). These data indicate that M2 (T70/T74), and M4 (T235/T239) together are the critical and may function synergistically to facilitate phosphorylation by ASK1.

ASK1 augments Siah1 levels and GAPDH-Siah1 binding in cells—We next addressed whether ASK1 modulates GAPDH-Siah1 signaling. Thus, we introduced wild-type (WT) ASK1, kinase-dead (KD) ASK1 that was generated with one amino acid substitution at 709 (K709M), and constitutively active (CA) ASK1 (aa 649-1375) in cells, respectively (41). We then examined how these distinct forms of ASK1 affected GAPDH-Siah1 binding. We previously reported that sodium nitroprusside (a nitric oxide donor) could affect GAPDH-Siah1 binding by *S*-nitrosylation of GAPDH (21), which was used as a reference of the binding change. Introduction of WT ASK1 dramatically augmented GAPDH-Siah1 binding, which was considerably greater than the change elicited by sodium nitroprusside in total cell lysates (Fig. 5*A*, *lower panel*). When kinase activity of ASK1 was selectively reduced (KD ASK1), such augmentation was also reduced. Consistent with this observation, introduction of CA ASK1 also dramatically augmented GAPDH-Siah1 binding (Fig. 5*A*, *upper panel*). Augmention of Siah1 levels was observed in an ASK1 kinase dependent manner (Fig. 5*A*). To ascertain that augmentation of GAPDH-Siah1 binding was indeed regulated by ASK1 activity we pretreated HEK293 cells with NQDI-1, a specific ASK1 inhibitor, before exposing cells the H_2O_2. Inhibition of ASK1 reduced endogenous GAPDH-Siah1binding induced by

H_2O_2 (Fig. 5*B*). These results suggest that ASK1, especially its kinase activity, is critical for augmenting Siah1 levels and GAPDH-Siah1 protein binding.

ASK1-induced GAPDH-Siah1 binding in cells is independent of p38 and JNK signaling—We considered the possibility that increased GAPDH-Siah1 binding could be affected by JNK and p38, two key kinases downstream of ASK1 (27). To test this idea, we used specific kinase inhibitors (SB203580 specific for p38 and SP600125 specific for JNK) and examined the effects on GAPDH-Siah1 binding in the presence of exogenous WT ASK1. Neither SB203580 nor SP600125 affected ASK1-Siah1, ASK1-GAPDH or GAPDH-Siah1 binding (Fig. 6*A*, *B*), suggesting that ASK1-induced GAPDH-Siah1 binding occurs independent to p38/JNK signaling.

ASK1 augments nuclear translocation of GAPDH and p300 acetylation in cells, which is likely to be affected by ASK1 phosphorylation on Siah1—The major downstream event of activated GAPDH-Siah1 stress signaling is nuclear translocation of the GAPDH-Siah1 protein complex (21). Thus, we questioned whether ASK1 might facilitate this nuclear translocation, and tested the effects of WT or KD ASK1. We observed robust nuclear translocation of the GAPDH-Siah1 complex in the presence of exogenous WT ASK1, whereas introduction of KD ASK1 did not elicit significant levels of GAPDH-Siah1 nuclear translocation (Fig. 7*A*).

We further tested whether this translocation by ASK1 was related to phosphorylation of Siah1. In the presence of ASK1 the complex of GAPDH and mutant Siah1 (M2+M4 mutant; lacking ASK1 phosphorylation sites) displayed significantly

reduced nuclear translocation compared to the complex of WT Siah1 and GAPDH (Fig. 7*B*).

We then questioned whether these Siah1 mutations (M2+M4) critically affect nuclear functions in ASK1-triggered GAPDH-Siah1 stress signaling. Thus, we examined GAPDH-p300 binding and acetylation of p300, which have been established as good functional indicators for nuclear GAPDH (22). Western blot analysis revealed a significant decrease in p300-GAPDH binding and acetylation of p300, which can be interpreted as a key consequence of reduced nuclear translocation of GAPDH in the presence of the M2M4 Siah1 mutant (Fig. 7*C*). These results suggest that phosphorylation of Siah1 by ASK1 is likely to play a key role in the GAPDH-Siah1 signaling cascade and subsequent functional effects in the nucleus.

DISCUSSION

Cellular signaling in response to stressors is crucial in homeostatic control and survival of all organisms. Thus, crosstalk among signaling cascades and their finely tuned regulation are expected. In the present study, we show that ASK1, a representative stress kinase, regulates GAPDH-Siah1 signaling (Fig. 8). Here we induce oxidative stress with H_2O_2, a known physiological and pathophysiological stressor, to demonstrate that ASK1, Siah1, and GAPDH form a ternary protein complex that is augmented under oxidative stress and is likely to be the basis of this signal network. We have identified Siah1 is a novel substrate of ASK1, with T70/T74 and T235/T239, as the critical phosphorylation sites on Siah1, at least *in vitro*. Phosphorylation by ASK1 was found to increase GAPDH-Siah1 binding, its nuclear translocation, and subsequent acetylation of nuclear p300.

Thus far, a specific posttranslational modification of GAPDH (*S*-nitrosylation/oxidation at C150) had been established as an initial trigger of GAPDH-Siah1 signaling (21). In the present study we show that specific posttranslational modifications of Siah1, the other partner of this complex, can also be a trigger of this cascade. ASK1 is activated in response to various stressors, such as oxidative stress and endoplasmic reticulum stress, it is likely that Siah1 is subsequently phosphorylated and mediates stress signaling by forming a complex with GAPDH. Thus, this study establishes the notion that the GAPDH-Siah1 cascade is activated by more than one mechanism in the presence of various cellular stressors. This cascade is also inhibited by at least two mechanisms: both interaction with a cytosolic protein GOSPEL (49) and a set of deprenyl-related compounds (42). It is likely that the GAPDH-Siah1 cascade, which is

crucial for homeostatic control, has multiple mechanisms that regulate its initiation in both positive and negative ways. It will be interesting to clarify how these two distinct posttranslational modifications (oxidation of GAPDH and phosphorylation of Siah1) are coordinated under different types of stress. Given that both ASK1 and GAPDH-Siah1 cascades are major routes of stress signaling, an important future question is to understand how GAPDH-Siah1 can also influence ASK1.

Here we demonstrate that ASK1 and GAPDH-Siah1 co-mediate stress signaling and activate p300. It is known that p300 has multiple functions in different conditions: for example, in the heart p300 activation can lead to heart hypertrophy mediated by myocyte enhancer factor-2 (MEF2) (50), whereas in the brain p300 affects memory function via cAMP response element-binding (CREB) (51). Roles for stressors in these conditions are appreciated, but further molecular mechanisms remain to be elucidated. Thus, the generation of new conditional knockout mice or inducible transgenic models targeting these molecules will be crucial to test context-dependent crosstalk of ASK1 and GAPDH-Siah1. As far as we are aware, such mice are not available at present. Better understanding of the crosstalk between these two stress cascades (ASK1 and GAPDH-Siah1) may provide a more integrated and comprehensive picture of how our body responds to stressors in a context-dependent fashion and how disturbances of such mechanisms may lead to pathological conditions.

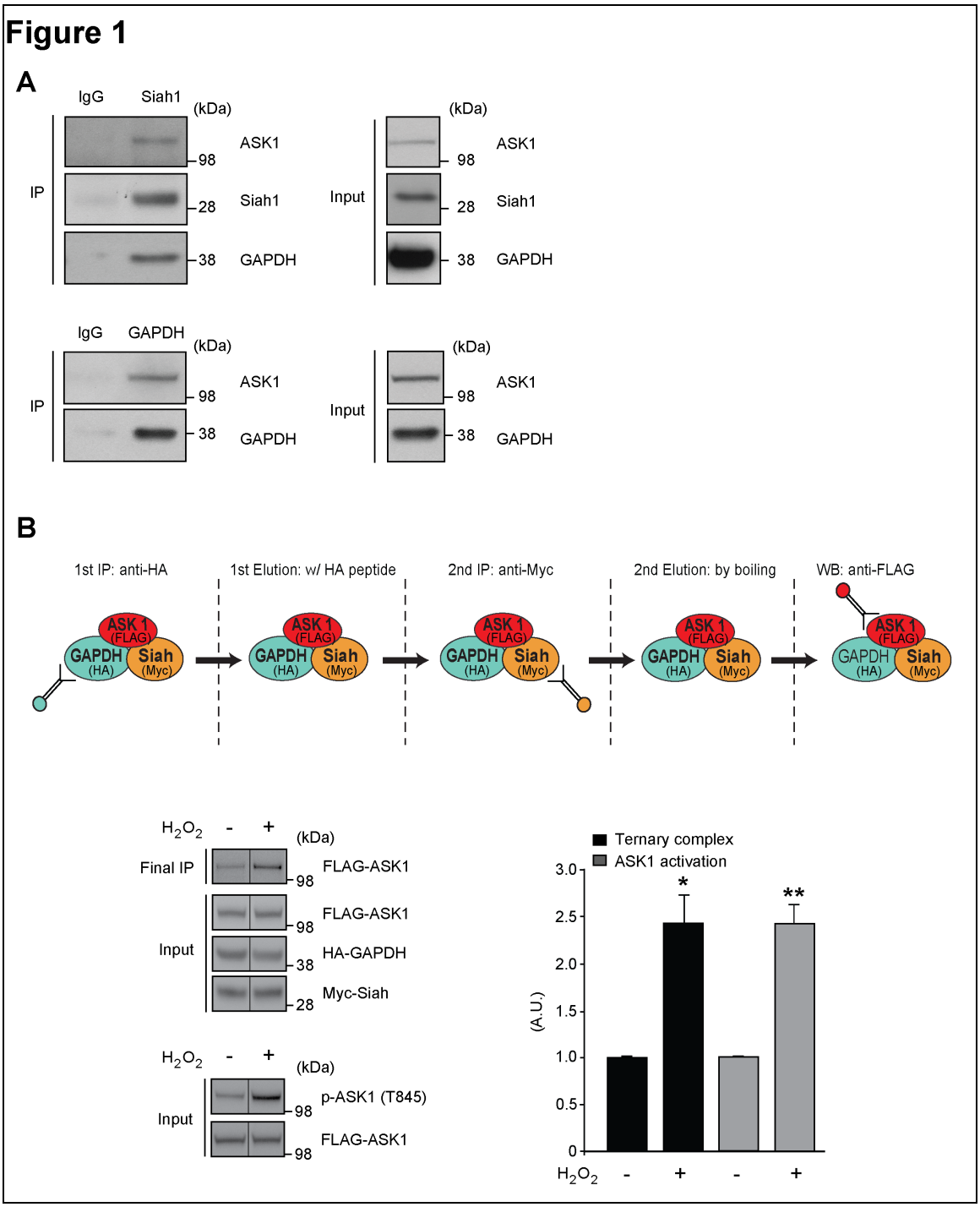

Figure 1. Siah1 and GAPDH bind with ASK1 and form a ternary complex in cells. (A) ASK1-Siah1 and ASK1-GAPDH binding in mouse brain extracts. Mouse brain extracts were immunoprecipitated (IPed) with anti-Siah1 or anti-GAPDH antibodies and analyzed by Western blot with anti-ASK1, GAPDH, and Siah1 antibodies. Input is total cell lysates for IP. (B) HEK293 cells expressing FLAG-ASK1, HA-GAPDH and Myc-Siah1 were treated with 1mM H_2O_2 for 30 min to induce activation of ASK1 (lower panel). Cell lysates were IPed in sequence with anti-HA and anti-Myc antibodies, respectively, and analyzed by Western blot with an anti-FLAG antibody (upper panel). Input is the starting material for IP. Ternary complex formation was quantified by densitometric analyses (t-test, *$p<0.05$, **$p<0.01$ versus no treatment FLAG-ASK1).

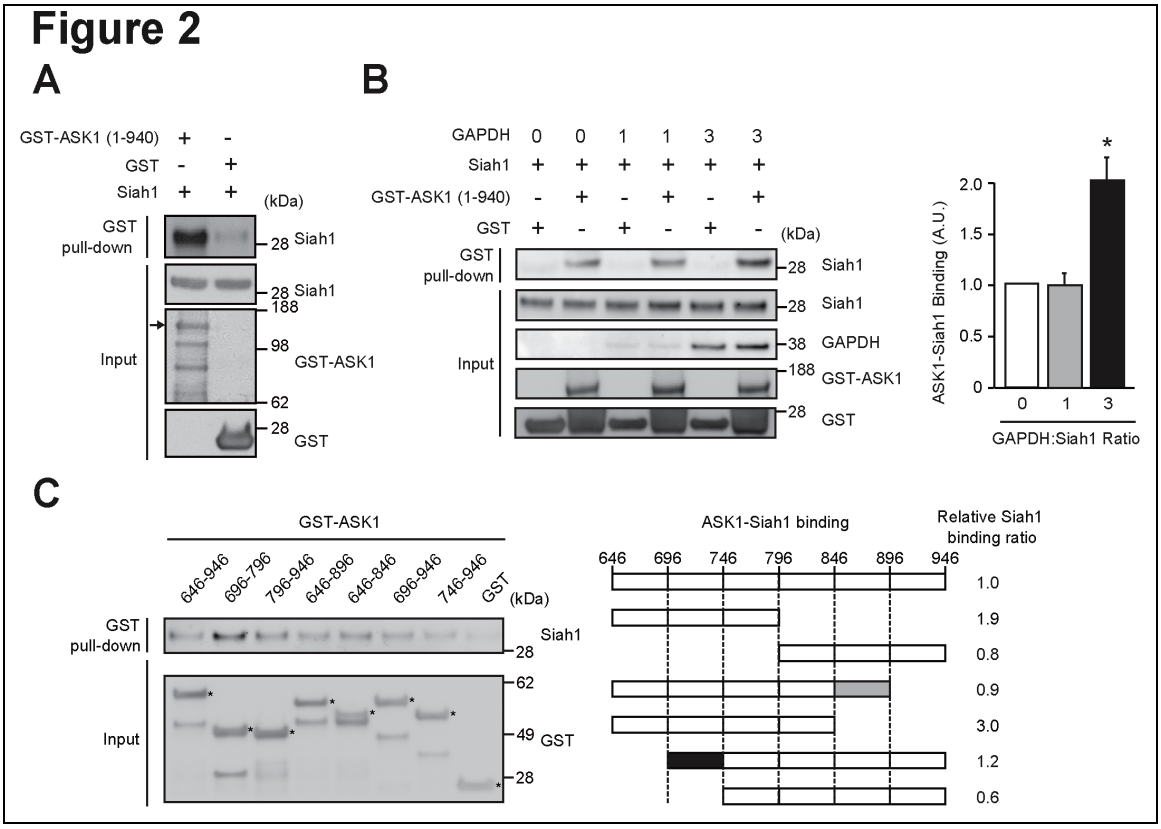

Figure 2. ASK1 directly binds to Siah1 and is modulated by GAPDH *in vitro*. (A) Recombinant GST-ASK1 [amino acids (aa) 1-940] or GST were incubated with recombinant Siah1 *in vitro* and subjected to GST pull-down, followed Western blot with an anti-Siah1 antibody. Input is the starting material for IP. Arrow indicates GST-ASK1 (aa 1-940). (B) GAPDH facilitates ASK1-Siah1 binding *in vitro*. ASK1-Siah1 binding was assessed by incubating recombinant GST-ASK1 (aa 1-940), or GST with recombinant Siah1 and GAPDH protein at 0, 1 or 3 (GAPDH:Siah1) molar concentrations, followed by to GST pull-down. Precipitates were analyzed Western blot with an anti-Siah1 antibody. Input is the starting material for IP. ASK1-Siah1 binding was quantified by densitometric analyses (t-test, *$p<0.05$ versus Siah1 (without GAPDH)). (C) Siah1 directly binds ASK1 within the kinase domain. Recombinant GST-ASK1 fragments of the kinase domain or GST were incubated with recombinant Siah1 *in vitro* and subjected to GST pull-down, followed by Western blotting with an anti-Siah1 antibody. Input is the starting material for IP. Numerical values indicate ASK1-Siah1 binding quantified relative to GST-ASK1 (aa 646-946). Within domain map black indicates critical binding domain and grey indicates potential inhibitory binding domain.

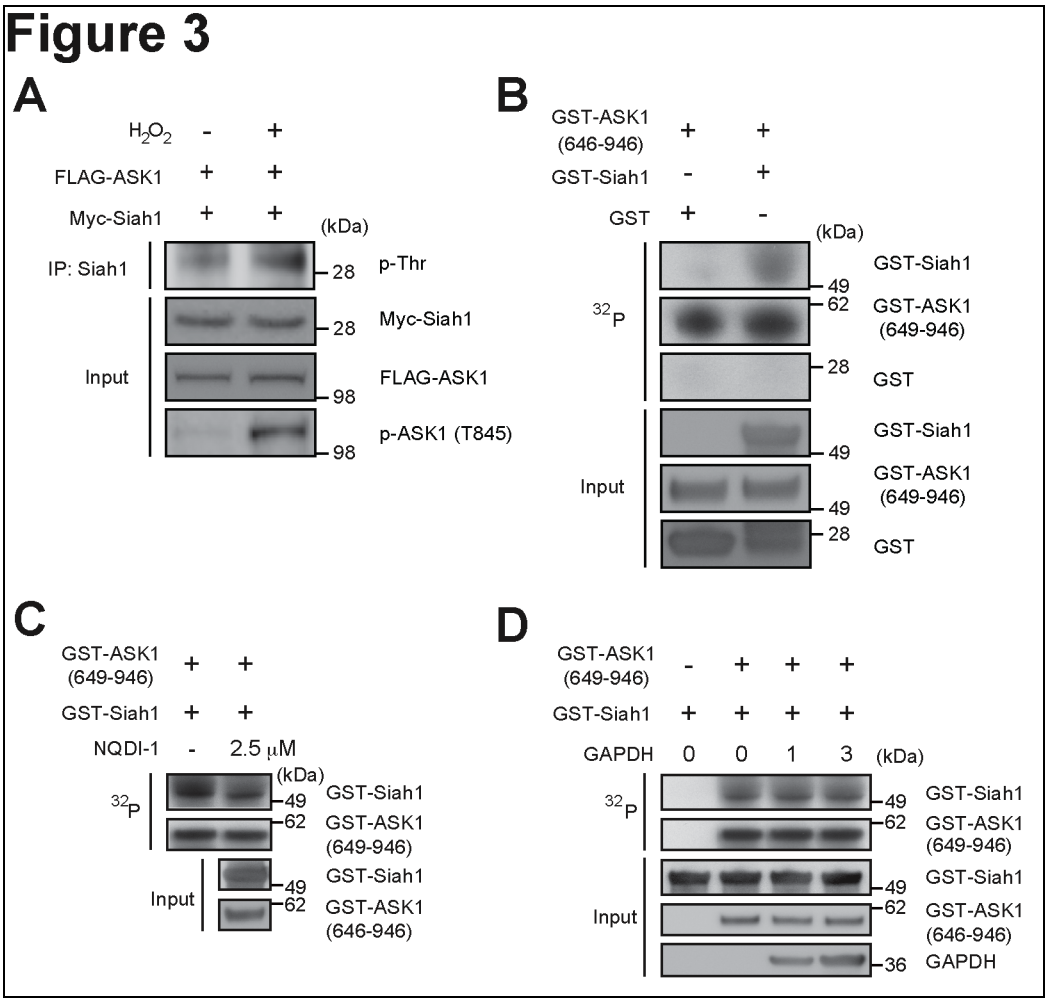

Figure 3. ASK1 phosphorylates Siah1 in cells and *in vitro*. (A) Stress-induced phosphorylation of Siah1 in cells. H2O2 treatment induced ASK1 activity and Siah1 phosphorylation. (B) Phosphorylation of Siah1 by ASK1 *in vitro*. (C) ASK1 inhibitor (NQDI-1) reduced direct Siah1 phosphorylation at both 2.5 μM and 25 μM concentrations. (D) In the presence of the ASK1 kinase domain GAPDH does not augment phosphorylation on Siah1. *In vitro* phosphorylation assays were performed by incubation of recombinant GST-Siah1 or GST with constitutively active GST-ASK1 (aa 646-946) in the presence of γ-^{32}P-ATP.

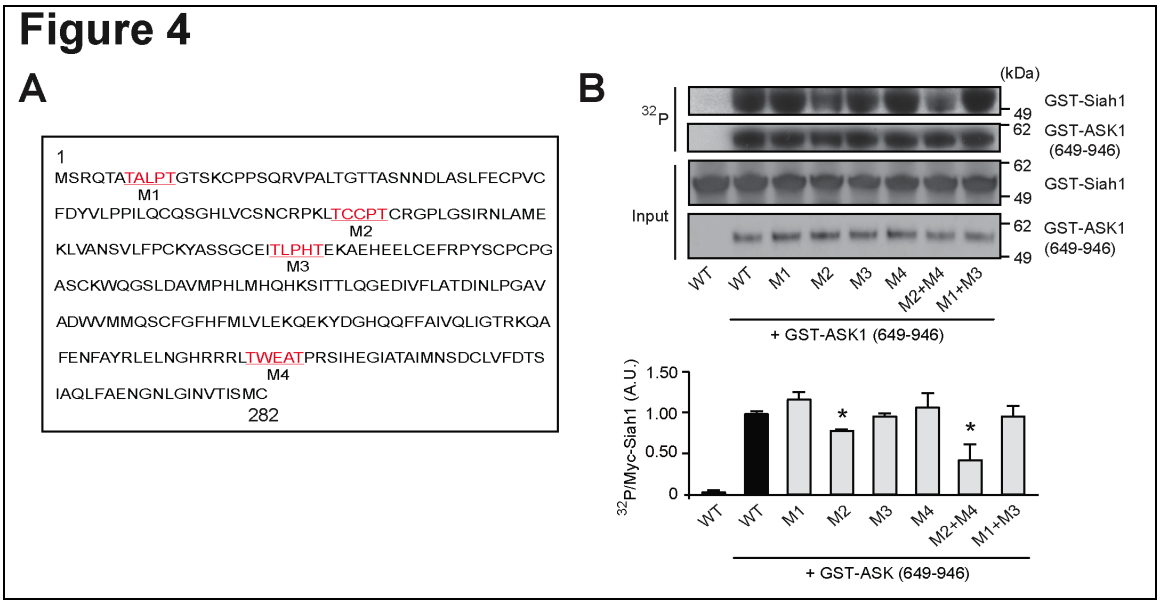

Figure 4. ASK1 phosphorylated Siah1 at specific phosphorylation motifs. (A) Four consensus (S/TxxxS/T) phosphorylation motifs for ASK1 in Siah1 protein: designated as M1, M2, M3 and M4. (C) M2+M4 mutant Siah1 shows a significant reduction of phosphorylation by ASK1 *in vitro*. Labeling with γ-^{32}P-ATP was carried out *in vitro* with recombinant GST-ASK1 (aa 646-946), along with several mutants of Siah1 (M1, M2, M3, M4, M2+M4 and M1+M3), followed by detection of Siah1 phosphorylation by autoradiography. Input is GST-ASK1 (aa 646-946) and Siah1 in starting material. Siah1 phosphorylation levels were quantified by densitometric analyses (t-test, *p<0.05 versus WT Siah1).

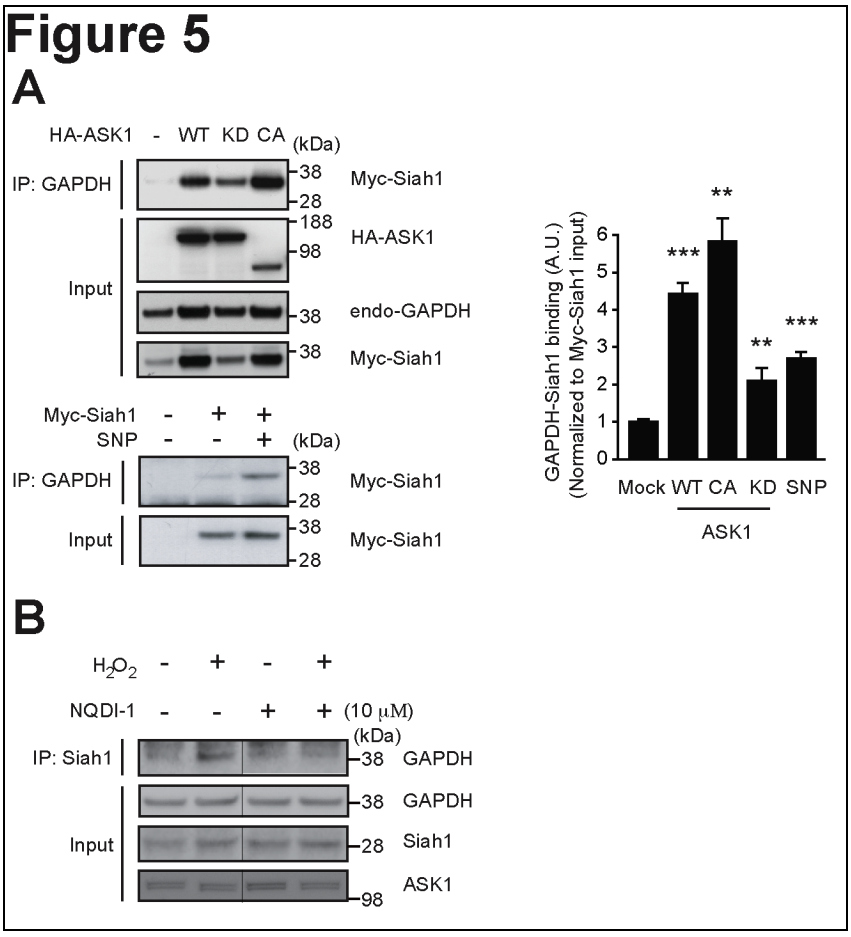

Figure 5. ASK1 facilitates GAPDH-Siah1 binding in cells. (A) Upper panel, ASK1 augments GAPDH-Siah1 binding in a kinase-dependent manner. Cell lysates of HEK293 cells expressing Myc-tagged wild-type (WT) Siah1 together with HA-WT ASK1, kinase-dead (KD) ASK1, or constitutively active (CA) ASK1, were IPed with an anti-GAPDH antibody and analyzed by Western blot with an anti-Myc antibody (for Siah1). Input is the total cell lysates. Lower panel, Sodium nitroprusside (SNP, a nitric oxide donor) elicits an augmented GAPDH-Siah1 binding. HEK293 cells expressing Myc-WT Siah1 were treated with SNP, cell lysates were IP with an anti-GAPDH antibody and immunoprecipitates were analyzed by Western blot with an anti-Myc (Siah1) antibody. Input is Myc-Siah1 in total cell lysates. Western blots were quantified by densitometric analyses (t-test, **$p<0.01$ and ***$p<0.001$ versus Mock). (B) ASK1 inhibitor NQDI-1 inhibits endogenous GAPDH-Siah1 binding. HEK293 cells were pretreated with 10 μM NQDI-1 for 1 h prior to treatment with 1 mM H_2O_2. Whole cell lysate were Iped with an anti-Siah1 antibody and analyzed by Western blot with an anti-GAPDH antibody. Input is the total cell lysates.

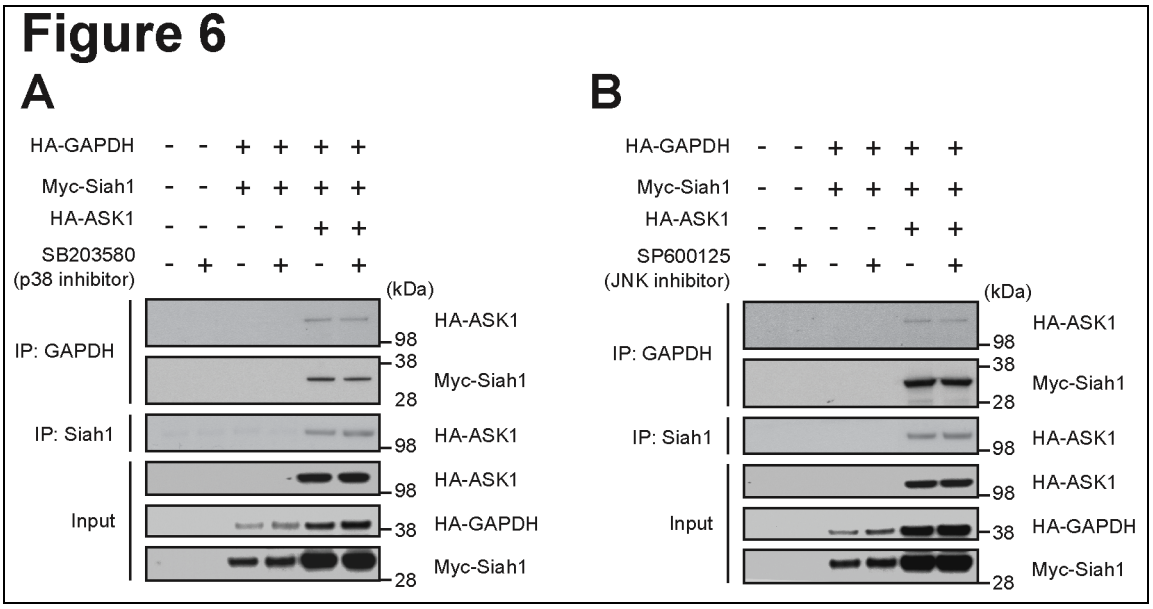

Figure 6. ASK1-Siah1, ASK1-GAPDH and GAPDH-Siah1 binding is independent of p38 and JNK signaling. ASK1-Siah1, ASK1-GAPDH and GAPDH-Siah1 binding occurs independent of p38 and JNK activation. (A) ASK1-Siah1, ASK1-GAPDH and GAPDH-Siah1 binding was assessed in lysates from HEK293 treated with 10 μM p38-specific inhibitor (SB203580) followed by IP and Western blot with indicated antibodies. Input is expression of HA-ASK1, HA-GAPDH and Myc-Siah1 in total cell lysates. (B) ASK1-Siah1, ASK1-GAPDH and GAPDH-Siah1 binding was assessed in HEK293 cells treated with 10 μM JNK-specific inhibitor (SP600125) followed by the IP and Western blot with indicated antibodies. Input is expression of HA-ASK1, HA-GAPDH and Myc-Siah1 in total cell lysates.

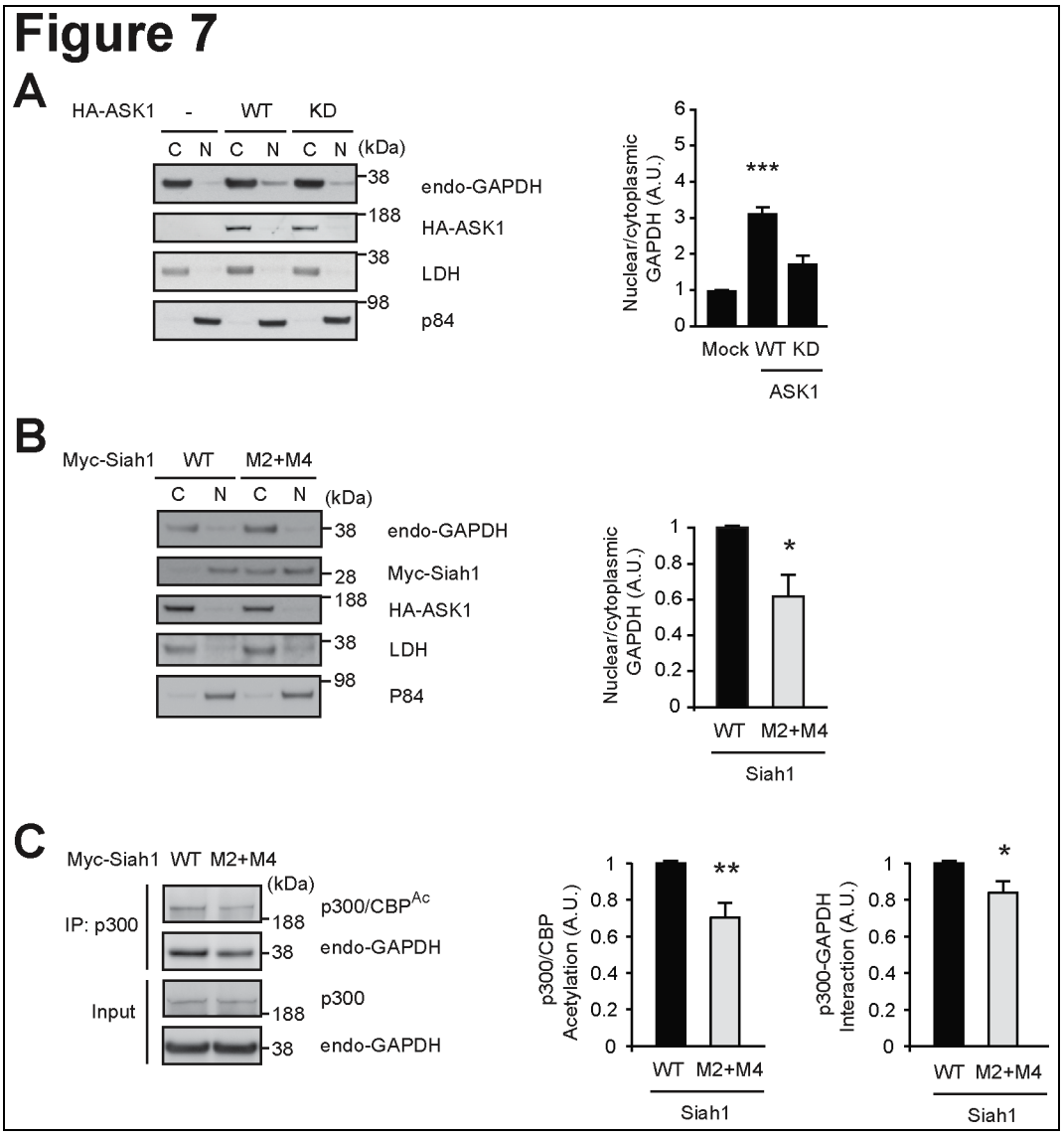

Figure 7. ASK1 augments nuclear translocation of GAPDH and p300 acetylation in cells, likely being mediated by ASK1 phosphorylation on Siah1. (A) ASK1 augments nuclear translocation of GAPDH in a kinase-dependent manner. Effects of ASK1 kinase activity on nuclear translocation of GAPDH were examined in HEK293 cells expressing HA-tagged WT or KD ASK1, followed by cellular fractionation and Western blot quantification of GAPDH nuclear/cytoplasmic ratios. GAPDH signals were normalized by LDH (a cytoplasmic marker) and P84 (a nuclear marker) (t-test, ***p<0.001 versus Mock). (B) M2 and M4 phosphorylation sites of Siah1 by ASK1 are critical to induce nuclear translocation of GAPDH. HEK293 cells expressing HA-WT ASK1 together with Myc-WT or M2+M4 mutant Siah1 were fractionated, and GAPDH nuclear/cytoplasmic ratios in each subcellular fraction were quantified (*p<0.05 versus WT Siah1). (C) M2 and M4 phosphorylation sites of Siah1 by ASK1 are required for acetylation of p300 and p300-GAPDH interaction. Lysates of HEK293 cells expressing HA-WT ASK1 together with Myc-WT Siah1 or M2+M4 mutant Siah1 were IP-ed with an anti-p300 antibody and analyzed by Western blot with acetyl-p300-Lys1499 / acetyl-CBP-Lys 1535 (p300/CBP^{Ac}) and GAPDH antibodies. Input is endogenous p300 and GAPDH in total cell lysates. Western blots were quantified by densitometric analyses (t-test, *p<0.05 and **p<0.01 versus WT Siah1).

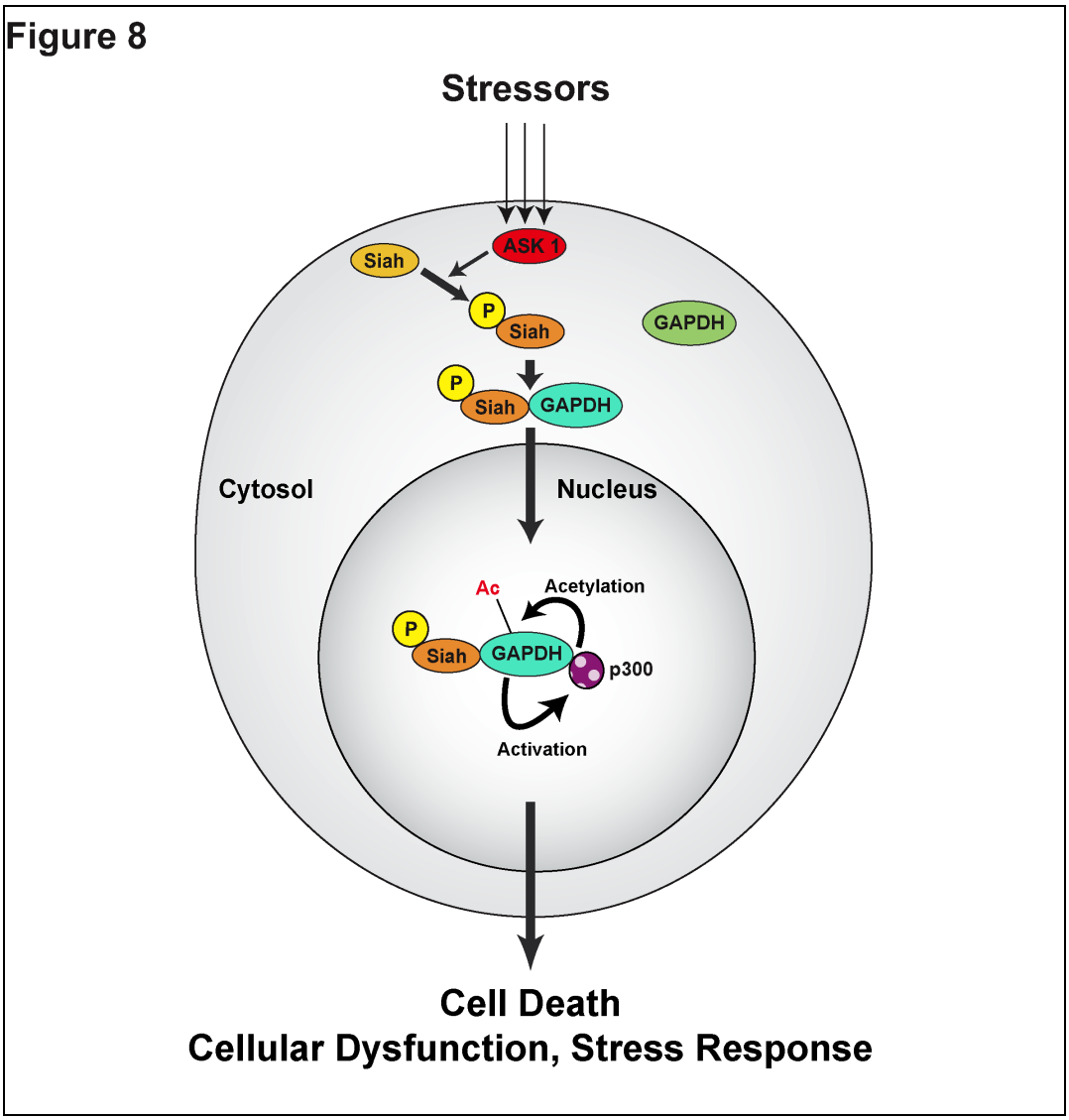

Figure 8. ASK1 regulates the GAPDH-Siah1 stress-signaling cascade. Exogenous stressors activate ASK1 kinase activity, ASK1 phosphorylates Siah1, phosphorylation of Siah1 induces GAPDH-Siah1 binding, the GAPDH-Siah1 complex translocates into the nucleus, in the nucleus GAPDH induces acetylation of p300.

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Chapter III: Conclusion

In this study we have identified ASK1 as a novel regulator of the GAPDH-Siah1 stress-signaling cascade. We found endogenous ASK1-GAPDH, ASK1-Siah1 and Siah1-GAPDH binding in adult mouse brain lysates. We show that upon treatment with oxidative stress these protein-protein interactions are mediated by formation of a ternary complex among ASK1, Siah1 and GAPDH. Further characterization of these protein interactions demonstated direct binding between ASK1 and Siah1, which was further augmented by GAPDH. Although direct ASK1-GAPDH binding was not observed *in vitro*, the observed augmentation in ASK1-Siah1 binding in the presence of GAPDH suggests that GAPDH may function as a scaffolding molecule between ASK1 and Siah1. Furthermore, direct interaction between the kinase domain of ASK1 and Siah1 suggested that Siah1 might be a substrate of ASK1. Phosphorylation studies and *in vitro* kinase studies demonstrate that stress-induced activation of ASK1 led to increased phosphorylation of Siah1 and that the ASK1 kinase domain directly phosphorylates Siah1. A specific inhibitor of ASK1 (NQDI-1) is shown to decrease Siah1 phosphorylation and further demonstrates that ASK1 induces direct phosphorylation of Siah1. Since GAPDH is capable of increasing ASK1(1-940)-Siah1 binding, but not Siah1 phosphorylation in the presence of the ASK1 kinase domain (646-946), we hypothesized that a region within the 1-646 domain is essential for GAPDH interaction. Siah1 is identified as a novel substrate of ASK1 and the M2 and M4 sites on Siah1 are demonstrated as two critical sites for phosphorylation by ASK1. We found GAPDH-Siah1 binding increases in an ASK1 kinase-dependent manner and that ASK1 is a stronger trigger of GAPDH-Siah1 binding than the previously reported S-nitrosylation of GAPDH. Cellular fractionation studies demonstrate that nuclear translocation of

GAPDH is augmented by ASK1 and that translocation of GAPDH is reduced in the presence of the M2M4 mutant Siah1. In the nucleus GAPDH was demonstrated to induce acetylation of transcriptional co-activator CBP/p300, which was also reduced in the presence of the M2M4 mutant Siah1.

Recognition of ASK1 as an activator of the GAPDH-Siah1 stress-signaling cascade identifies ASK1, and the M2M4 sites on Siah1, as novel therapeutic targets to reduce nuclear translocation of GAPDH. ASK1 activation has predominantly been associated with signaling cascades that ultimately lead to cell death. However, recent studies have reported that activation of ASK1 promotes differentiation and survival of neuronal and non-neuronal cells (1-3). Similar to GAPDH, ASK1 may have opposing functions that simultaneously regulate cell survival and cell death. While inhibitors such as NQDI-1 may inhibit GAPDH-Siah1 stress signaling, global inhibtion of ASK1 could have detrimental side effects. Therefore, developement of molecules that inhibit ASK1-induced GAPDH-Siah1 stress-signaling by targeting the M2M4 sites might be a valuable and more specific therapeutic strategy against diseases such as, cancer, cardiovascular disease, neurodegeneration, ischemia and major mental illness.

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